ESCHERICHIA COLI, POULTRY, AND PRECIPITATION: A WATERSHED STORY

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ABSTRACT

Ryan Eric Leighton: *Escherichia coli*, Poultry, and Precipitation: A Watershed Story
(Under the direction of Jill Stewart)

Industrial poultry production in concentrated animal feeding operations (CAFOs) in North Carolina has grown rapidly, with North Carolina now ranked nationally as a top poultry producer. The Yadkin-Pee Dee River Basin harbors the highest density of poultry production among North Carolina river basins. This study examined the effect of poultry CAFOs on water quality in the Yadkin-Pee Dee River Basin by comparing watersheds with CAFOs (n=5) to those without CAFOs (n=4). In partnership with Yadkin Riverkeeper, we collected surface water samples during both dry weather and wet weather events, for a total of 36 samples analyzed. Each sample was evaluated for *Escherichia coli* concentrations and antibiotic resistance profiles. Few *E. coli* isolates were antibiotic resistant. A multiple linear regression indicated presence of poultry CAFOs resulted in higher *E. coli* concentrations compared to background sites and that every 1-mm increase in precipitation resulted in higher *E. coli* concentrations.

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LIST OF ABBREVIATIONS AND SYMBOLS

AFO	Animal Feeding Operation
BK	Background
CAFO	Concentrated Animal Feeding Operation
CFU	Colony Forming Unit
CI	Confidence interval
DL	Detection limit
E. coli	*Escherichia coli*
FIB	Fecal Indicator Bacteria
FIO	Fecal Indicator Organism
Km	Kilometer
mL	Milliliter
MLR	Multiple Linear Regression
P	Poultry
P	P-value
PCR	Polymerase Chain Reaction

CHAPTER 1: INTRODUCTION

A watershed is an area of land that drains rainwater or snow into a body of water like a stream, river or lake (United States Geological Survey, 2016). These surface waters make up about 80% of the water that is used daily and are important for drinking water, agriculture, and habitat for various plants and animals (National Ground Water Association, 2012). However, runoff pollution can degrade the quality of the watershed. Precipitation exacerbates runoff pollution by collecting various pollutants in surfaces and soils of the watershed and concentrating them in the surface waters (Parker et al., 2010). Animal waste is a contributor to surface water pollution, and concentrated animal feeding operations (CAFOs) can be major contributors to water pollution if waste treatment is not handled properly (Mallin & Cohoon, 2003).

Concentrated animal feeding operations (CAFOs) can house thousands of animals for food production, and these operations generate significant volumes of waste that require management and disposal (Hribar, 2010). There are currently an estimated 50 million chickens in the upper Yadkin-Pee Dee River Basin and these chickens collectively produce 1,000,000 tons of manure each year according to data from NRAES (1999) and Barker (1990) and calculations from Chastain et al. (2000) (Yadkin Riverkeeper). With poultry CAFOs, practices like reusing the waste for fertilizer for crops can lead to leaching of manure into watersheds, degrading the water quality (Burkholder et al., 2006; Fisher et al., 2005). Many microorganisms that can exist in watersheds from fecal runoff are sources of potential human health hazards, which is why tracking and monitoring fecal indicator organisms is of great public health importance.

Tools have been developed to track fecal contamination in the environment, including the use of fecal indicator bacteria. *Escherichia coli*, a type of fecal coliform bacteria that is found in the intestines of humans and animals, is commonly used as an indicator of human and/or animal fecal contamination in fresh water (Pitout et al., 2017; Centers for Disease Control and Prevention, 2018). *E. coli* is monitored as its presence is indicative of other potential pathogenic microbes that could be present in water (Edberg et. al., 2000). Due to antibiotic use in animal agriculture, monitoring antibiotic resistant *E. coli* is also of interest (Gustafson & Bowen, 1997). Antibiotic use remains common in food animal production and antimicrobial resistance from agricultural practices is a potential public health problem as humans can be exposed to antibiotic resistant pathogens by consumption of domesticated farm animals or coming into contact with the pathogens in the environment (Silbergeld et al., 2008). These resistant bacteria in animal waste, including *Escherichia coli*, can also end up in watersheds from runoff, and concentrations can potentially increase during precipitation events (Campagnolo et al., 2002: Harris et al., 2018; Hill et al., 2005).

Precipitation can further exacerbate microbial pollution by collecting and draining the microorganisms into the nearest body of water. Previous studies have shown microbial concentrations were directly correlated with increased rainfall and streamflow (Lipp et. al, 2001; Shehane et al., 2005). A study conducted by Noble et al. (2003) also found *E. coli* concentrations greatly increased after heavy precipitation events. However, many public health studies have focused on beach water quality after storm events, which leaves the potential impacts of precipitation on many inland watersheds unknown. Tornevi et al. (2014) found that rainfall led to higher concentrations in a freshwater river and concluded that precipitation is a main contributor to fluctuating water quality. The location of poultry CAFOs in watersheds and near creeks, streams, rivers or lakes can make these bodies of water vulnerable to microbial pollution, especially during heavy precipitation events (Wing et al., 2002).

Studies and reviews have been conducted to examine how poultry CAFO waste can affect the chemical and biological parameters of water quality by nutrient and pathogen contamination. Nutrients such as nitrogen and phosphorus have been found in large quantities in poultry waste, with chicken waste having the highest amount of nitrogen and phosphorus of animal waste (Mallin and Cahoon, 2003; Martin and Gershuny, 1992). Poultry waste runoff can lead to influxes of nitrogen and phosphorus into a body of water, causing eutrophication. Eutrophication, along with warmer temperatures, encourages algal bloom growth, causing removal of oxygen from the water (hypoxia) as the algae grow exponentially, thus reducing water quality. Human zoonotic pathogens have been found in chicken litter, and human health can be negatively impacted by ingestion of pathogens from this litter from contaminated recreational or drinking-waters, with symptoms such as abdominal cramps, bloody diarrhea and vomiting (Rogers and Haines 2005; Craun et al. 2010; Dale et al. 2010; USEPA 2013; Mayo Clinic, 2018). North Carolina specifically is one of the top producing poultry states in the United States, and the impact of its poultry CAFOs on microbial water quality and how precipitation can affect watershed water quality needs to be assessed.

North Carolina is ranked nationally as the number four broiler chicken producer, and number three in total poultry production (North Carolina Poultry Federation, 2018). The upper Yadkin-Pee Dee River Basin has the highest poultry production in the state, with an estimated 50 million chickens (Yadkin Riverkeeper). These chickens collectively produce an estimated 1,000,000 tons of manure each year, which consequently can leach into the Yadkin-Pee Dee River Basin and surrounding environment in North Carolina, potentially decreasing water quality (Environmental Working Group, 2016). While several studies have examined fecal contamination from CAFOs (Campagnolo et al., 2002; Burkholder et al., 2007), including in eastern North Carolina's coastal basin and have considered effects of storm events (Burkholder et al., 1997; Mallin, 2000; Mallin et al., 1999), there has been a lack of research into poultry CAFOs affecting water quality in western North Carolina, specifically in the Yadkin-Pee Dee River

Basin, which is located largely in the piedmont region and is more susceptible to increased surface water runoff and erosion than the coastal region (Markewich et al., 1990). This study will address that gap in knowledge.

This research examines the *E. coli* concentrations and antibiotic resistance profiles in the Yadkin-Pee Dee River Basin in North Carolina and tests how precipitation events can influence *E. coli* concentrations. The purpose of this study was to compare microbial water quality in watersheds that had poultry CAFOs upstream to background sites that lacked poultry CAFOs by assessing *E. coli* concentration and antibiotic resistance following dry periods and precipitation events. Validation of CAFO contamination during precipitation events could inform regulatory bodies like the state department of natural resources and the United States Environmental Protection Agency to increase their efforts in helping maintain water quality of environmental waters.

CHAPTER 2: OBJECTIVES

1. Measure and compare *E. coli* concentration in surface water sites with and without upstream poultry CAFOs.

2. Measure and compare the prevalence of antibiotic resistant *E. coli* in surface water sites with and without poultry CAFOs.

3. Evaluate the effect of precipitation on *E. coli* concentrations in the upper Yadkin-Pee Dee Watershed.

CHAPTER 3: REVIEW OF LITERATURE

Introduction

Fecal contamination of bodies of water can lead to waterborne illnesses and is detrimental to human health, with microbial contamination being a major cause. Concentrated animal feeding operations (CAFOs) are a potential culprit to microbial water contamination, as animal waste from these operations can end up in streams, rivers and lakes, especially from rain events (Hribar, 2010). Fecal indicator bacteria (FIB) like *Escherichia coli* have been used to detect and determine the level of fecal contamination in environmental waters to protect the general population from water-related pathogens (USEPA 2006; USEPA Office of Water 2015). However, due to antibiotic use to protect animals from infection, CAFOs have consequently created antibiotic resistant bacteria including *E. coli*, which also can end up in the environment from animal waste (Hribar, 2010). One animal production industry, poultry, is of concern. The poultry industry in North Carolina is booming, with North Carolina being ranked nationally as the number four broiler chicken producer, and number three in total poultry production (North Carolina Poultry Federation, 2018). The upper Yadkin-Pee Dee River Basin has the highest poultry production in the state, with an estimated 50 million birds in 2014 (Yadkin Riverkeeper). The goal of this review is to discuss existing literature on microbial water pollution from poultry CAFOs, including background of poultry CAFOs and their environmental health effects, *E. coli*'s role in monitoring water quality and the bacteria's potential of acquiring antibiotic resistance, precipitation's effect on water quality, and North Carolina's place in the poultry production industry.

Poultry concentrated animal feeding operations

American agriculture has transitioned from family-owned small farming to large-scale corporate farming in the last century, with a few companies now producing most of the food animals (Macdonald and McBride, 2009). Today, production of these animals occurs in CAFOs, which are essentially large-scale industrialized agricultural factory farms. To qualify as a CAFO, a farming operation must first be considered an animal feeding operation (AFO) which is defined as: "a lot or facility where animals are kept confined and fed or maintained for 45 or more days per year, and crops, vegetation, or forage growth are not sustained over a normal growing period" (Hribar, 2010; USEPA, 2009). The benefit of CAFOs stem from being the operations being well managed, located and monitored as they can reduce cost of animal production and thus reduce consumer cost by increased efficiency in feeding and housing (Hribar, 2010). CAFOs are classified by type and number of animals, and by how they discharge their animal waste into the nearest body of water. There are size thresholds in considering a CAFO to be small, medium or large. For poultry, especially laying hens or broilers, the CAFO, (which has a liquid manure handling system) is considered large with 30,000 or more chickens, medium with 9,000 – 29,999, and small with 9,000 or less chickens (USEPA, 2009). The large number of animals means a large amount of waste, which is where most of the environmental health issues arise.

Poultry CAFO waste can have several types of contaminants like nutrients, pathogens, and antibiotics (Hribar, 2009). Previous studies have shown that poultry CAFOs have contaminated surrounding watersheds by runoff containing poultry litter (bedding contaminated with feces) (Campagnolo et al., 2002; Mallin & Cohoon, 2003). Poultry litter can lead to degradation of water quality through chemical and microbial pollution. A study conducted by Harden (2015) found that watersheds that had swine and poultry CAFOs exhibited significantly greater nutrient contamination, including ammonium, nitrate and total N, compared to watersheds that lacked these operations. Stone et al. (1995) also found a stream that had both swine and poultry CAFOs had elevated nutrient concentrations

during both dry and wet weather events compared to a nearby background stream that lacked these operations. Nutrients in poultry waste like nitrogen and phosphorus can contribute to eutrophication, which is when there are excessive amounts of nutrients in a body of water, and this leads to algal bloom growth which can be detrimental to local ecosystems (Slonczewski, 2016; United States Environmental Protection Agency, 2017).

A study conducted by Mallin et al. (2015) found that in eastern North Carolina, watersheds that contained both swine and poultry CAFOs did not meet NC water quality standards (NCDENR, 1999), as they exceeded the fecal coliform standard's average of 200 CFU/100 mL for "5 consecutive samples during any 30-day period" and exceeded "400 CFU/100 mL in more than 20% of samples examined." A high concentration of CFU/100 mL points to possible pathogen presence, and pathogens indicated by *E. coli* can be harmful if ingested, potentially causing abdominal cramps, bloody diarrhea and vomiting (Mayo Clinic, 2018). Human zoonotic pathogens have been found in chicken litter, like *Escherichia coli* O157:H7, *Salmonella*, *Campylobacter*, *Cryptosporidium parvum*, and *Giardia lamblia*, and thus have been consequently found in runoff and in surrounding watersheds according to a review conducted by the United States Environmental Protection Agency (USEPA) in 2013. A study conducted by Claire Hruby in Iowa found that some of these pathogens from poultry manure can survive weeks in soil, which means heavy precipitation events could also cause water contamination by collecting and discharging the slurry (soil and water mixture) into the nearest body of water.

Antibiotics are found in residual levels in waste, as they are used to ensure the animals can stay healthy in fighting off potential infection and, until recently, to promote growth (Marshall and Levy, 2011; Gustafson & Bowen, 1997). However, overuse of antibiotics has led to antibiotic resistance in pathogens like *E. coli* due to selective pressure, causing some treatments for infection to be ineffective (Kaufman, 2000; Martinez, 2008). In 2013 the Food & Drug Administration (FDA) announced a plan to phase out certain medical antibiotics that were used in livestock to curb antibiotic resistance (FDA,

2013). Tyson, the leading poultry producer in the country, has curtailed antibiotic usage, and the company notably claimed it would eliminate antibiotics important to human medicine in raising its poultry by 2017 (Meyer, 2017). Monitoring *E. coli* and its potential for antibiotic resistance is helpful in maintaining water quality and subsequently public health.

E. coli and its role in water quality

Escherichia coli is a gram-negative, rod-shaped type of fecal coliform bacteria that is found in the intestines of humans and animals. Most strains are considered harmless to humans, but some strains produce Shiga-toxin, which can cause hemorrhagic diarrhea, and the serotype O157:H7 is the one most related to foodborne illnesses (The World Health Organization, 2018). *E. coli* is commonly used as a fecal indicator organism (FIO) of human and/or animal fecal contamination in fresh water (Pitout et al., 2017; Centers for Disease Control and Prevention, 2018). *E. coli* is monitored in water as its presence is indicative of other potential pathogenic microbes that could also be present (Edberg et. al., 2000). For *E. coli* to qualify as an FIO, it should ideally meet criteria according to The Routledge Handbook of Water and Health (Bartram et al. 2015). Some of the criteria include:

- Being present whenever enteric pathogens are present
- Occurring in greater numbers than pathogens
- Broad applicably and detectability in all types of water that humans may encounter
- Specific to a fecal source with humans or animal species that share fecal-oral pathogens with humans
- Being inexpensively, reliably, rapidly, and distinctly detectable
- Being randomly distributed in a sample

While no one fecal indicator organism currently satisfies all the criteria under all circumstances, many regulatory agencies and scientists still consider *E. coli* and members of the fecal coliform group as the

best for microbial water quality testing (Tipton, 2017; USEPA, 2009). Due to high volumes of antibiotics used in animal agriculture, monitoring antibiotic resistant *E. coli* is also of interest (Gustafson & Bowen, 1997).

Some poultry CAFOs use antibiotics to prevent or treat diseases and, until recently, to promote growth, but this can create potential antibiotic resistance in bacteria like *E. coli* due to selective pressure (Gustafson & Bowen, 1997; Martinez, 2008). Antibiotic resistance occurs when the antibiotic kills most of the intended susceptible bacteria, but a small number that are naturally immune to the antibiotic survive and reproduce, creating a population of predominantly antibiotic resistant bacteria (CDC, 2018). Antibiotic resistance has been an increasing public health issue in relation to CAFOs, as the use and overuse of antibiotics in animal feed has led to microbes becoming antibiotic resistant (Kaufman, 2000). Animals like poultry do not completely metabolize the antibiotics and can still exist in their waste (Hriibar, 2010). One study tested antibiotic resistance of *E.* coli in fecal samples of turkeys, broilers, their famers and their slaughterers, and found that there was statistically significantly higher ($p < 0.005$) antibiotic resistance to ciprofloxacin, flumequine and neomycin as compared to laying hens that didn't have high antibiotic usage (Boggard, 2001). A related study also found high prevalence of antibiotic resistance, but of *Staphylococcus aureus* in turkey (79%; 22/28) and chicken (26%; 6/23) isolates (Waters et al., 2011). Antibiotic resistance is not confined to poultry CAFOs but is a concern across industrial food animal production facilities. In 2007, Sapkota et al. (2007) analyzed surface water samples downstream of a swine CAFO and found statistically significant antibiotic resistance of erythromycin ($p = 0.02$) and clindamycin ($p < 0.001$) in enterococci. Christenson and Stewart (2018) also found higher antibiotic resistance in *E. coli* isolated from surface waters downstream of CAFOs compared to background watersheds (19% vs. 6%). Some studies have also noted higher levels of contamination following precipitation events.

Precipitation's effect on water quality

Precipitation can further exacerbate CAFO waste leaching and microbial pollution by collecting and dumping the waste and microorganisms into the nearest body of water. Studies have shown microbial concentrations are directly correlated with increased rainfall and streamflow (Lipp et al., 2001; Shehane et al., 2005). A study conducted by Noble et al. (2003) found *E. coli* concentrations greatly increased after heavy precipitation events. Tornevi et al. (2014) found that rainfall led to higher *E. coli* concentrations in a freshwater river and that precipitation drives water quality's variation. The location of poultry CAFOs in watersheds and near creeks, streams, rivers or lakes can make these bodies of water vulnerable to microbial pollution, especially during heavy precipitation events (Wing et al., 2002). High *E. coli* concentrations have also been found in the sediment of streams, rivers and lakes and a previous study found increased *E. coli* concentrations into the water column due to resuspension of these sediments from rainstorms. (Kim et al., 2010). This is consistent with research conducted by Cho et al. (2010) who observed sharp increases of fecal indictor bacteria after precipitation events from sediment.

Poultry Production in North Carolina

North Carolina is a top state in agricultural industry, with its poultry production being its leading agricultural income generator, making up ~ 40% of North Carolina's agricultural income. Poultry production has generated more than $34.4 billion for North Carolina's economy, and has created more than 100,000 jobs. North Carolina ranks 2nd in the United States in turkey, 4th in broiler chicken and 8th in egg laying chicken production (Caruthers, 2016). The upper Yadkin-Pee Dee River Basin has the highest poultry production in the state, with an estimated 50 million chickens according to Yadkin Riverkeeper. With all these chickens comes the amount of waste they produce, which is a mounting public health issue. These chickens collectively produce an estimated 1,000,000 tons of manure each year according to data from NRAES (1999) and Barker (1990) and calculations from Chastain et al. (2000). This poultry

manure consequently could leach into the Yadkin-Pee Dee River Basin and surrounding environment in North Carolina, potentially decreasing water and environmental quality downstream (Environmental Working Group, 2016).

CHAPTER 4: METHODS

Sample Collection

Water samples were collected in the Yadkin-Pee Dee River Basin from late October to mid-November in 2017. Each sample was collected in a sterile 1 L sample bottle that was triple-rinsed, then filled with the sample water and capped. Nitrile gloves were worn for each sample collection to ensure no cross contamination. After the bottle was filled and re-capped, the sample bottles were immediately stored in a cooler on ice and transported back to the laboratory in Chapel Hill for processing. All samples were processed within 24-hrs of sample collection.

Nine samples were collected for each of four sampling events by the Yadkin Riverkeeper, totaling 36 samples. Four of the nine samples (BK1-BK4; Figure 1) were taken from background sites that contained no CAFO upstream in the watershed, while the other 5 samples (Figure 1., P1-P5) were taken from watersheds that contained at least one poultry CAFO upstream based on Environmental Working Group (EWG) data for poultry barns (Environmental Working Group & Waterkeeper Alliance, 2016). Background sites had primarily agricultural land use in their upstream contributing watersheds, although these watersheds did not contain any type of CAFO based on EWG data or other known point sources of potential contamination, such as wastewater treatment plants (Environmental Working Group & Waterkeeper Alliance, 2016). Poultry sites are were situated in watersheds with a poultry CAFO located upstream in the watershed, and also did not have any other kind of CAFO or known point source.

Sample events on 10/24/17 (precipitation event A), 11/9/17 and 11/14/17 (precipitation event B) had measured precipitation in the two-days preceding sampling, and the last sampling event on 11/17/17 had no rainfall and is considered the closest to a baseline. Precipitation event A was the heaviest precipitation event (48 mm), with the 9 sample sites' rainfall averaged together. The sampling

event that occurred during event A was during peak rainfall of this sampling period, as is confirmed by rainfall data obtained from USGS 02111391 near Wilkesboro, NC (Figure 5A). Precipitation event B on 11/9/17 was a lighter precipitation event and sampling also occurred during its peak rainfall of this sampling period (Figure 5B). The rainfall event on 11/14/17 was the lightest event (Figure 5C). The sample event on 11/17/17 (Figure 5D) was taken with no two-day antecedent rainfall, which is why the event is considered closest to a baseline water/stream discharge level.

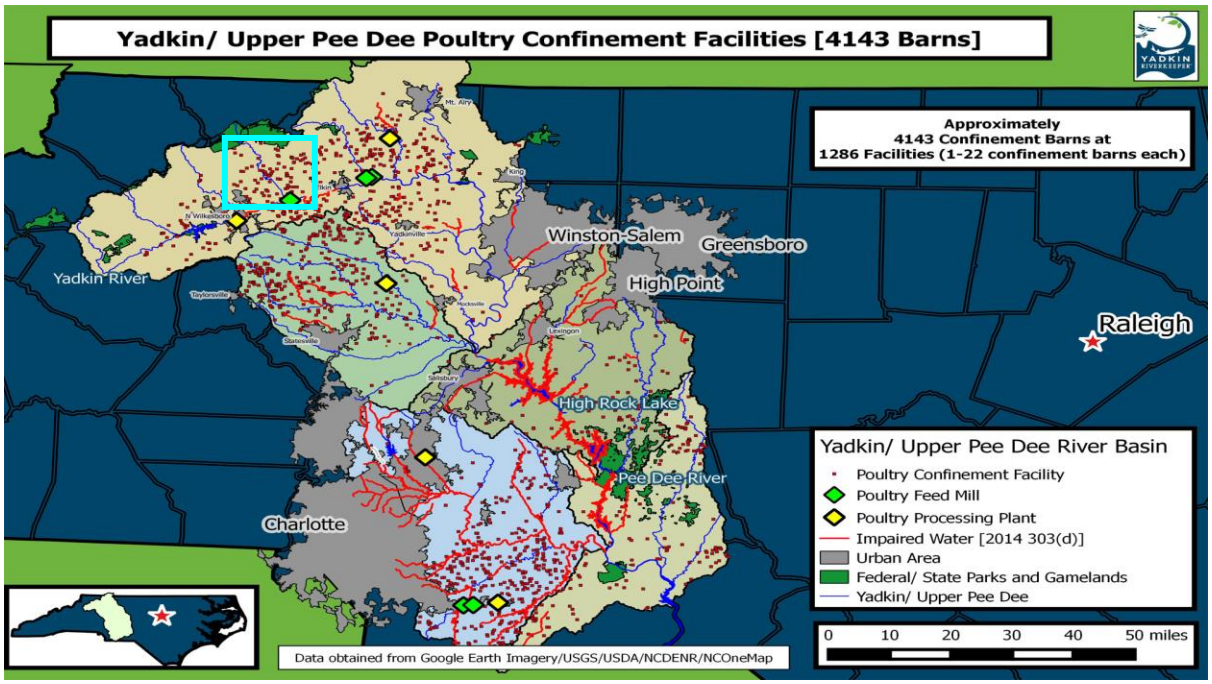

Figure 1. The Yadkin-Pee Dee River Basin in North Carolina with existing poultry operations in 2014. The light blue box on map is approximately where this study's sampling occurred.
Note: Map used in permission by Yadkin Riverkeeper.

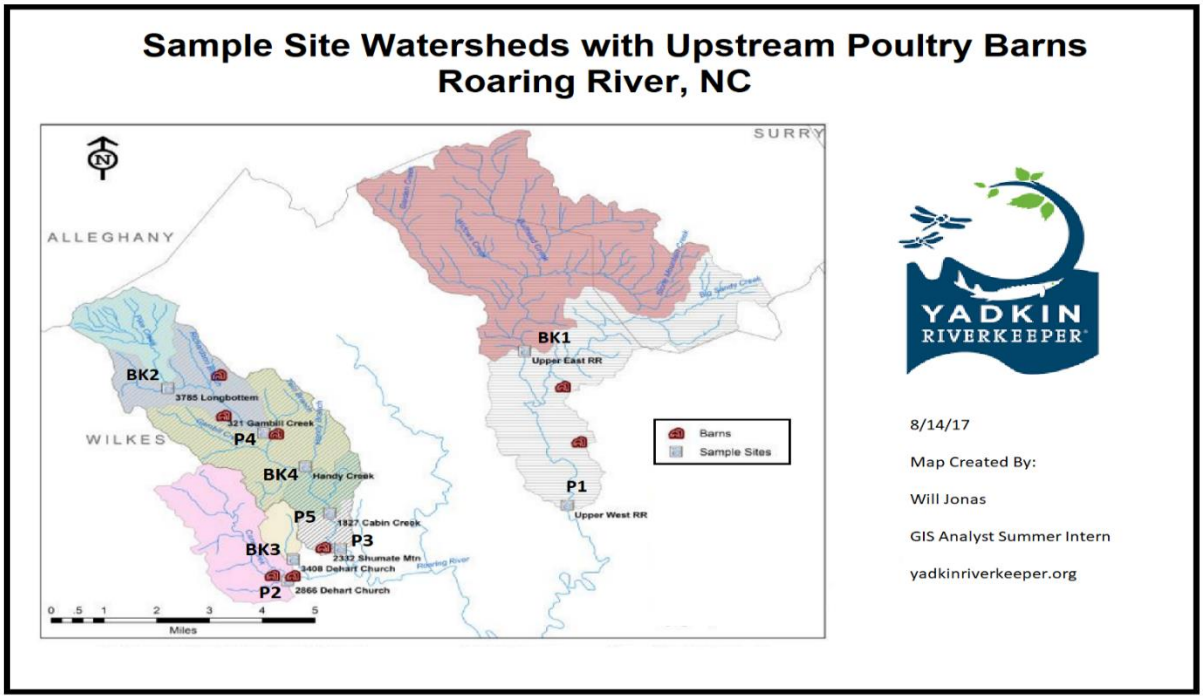

Figure 2. Sample locations (courtesy of Yadkin Riverkeeper). Barns refer to CAFO locations that house food animals.

Sample Processing

For every sample collected, standard membrane filtration methods were used to quantify concentrations of *E. coli* from each water sample collected (United States Environmental Protection Agency, 2002). Processing and filtration occurred within 1 day after each sampling event. 50mL of each of the 9 samples was filtered onto its own membrane and repeated for 25mL, 5mL, and 1mL volumes and aseptically placed on 50 mm plates containing selective mTEC media (Sigma-Aldrich). The mTEC plates were inverted and incubated at 44 ^{o}C for 22 hr (+/- 2 hr). Plates were examined for colonies with morphological characteristics of *E. coli* as per manufacturer's instructions. Plates that contained 20 to 80 colonies were counted, then summed and used to calculate concentrations of colony forming units (CFUs) per 100mL (United States Environmental Protection Agency, 2010). Up to five *E. coli* colonies per sample were then isolated, purified and confirmed through biochemical testing including indole production with Kovacs Reagent (Acharya, 2017).

Antibiotic Resistance Testing

Antimicrobial resistance testing was conducted on all archived *E. coli* isolates using standard Kirby-Bauer disc diffusion methods on Mueller Hinton II agar (Sigma Aldrich) and following standard Clinical Laboratory and Standards Institute (CLSI) guidelines (Clinical Laboratory Standards Institute, 2014). Isolates were tested for resistance to eleven antibiotics (Table 3) in different antibiotic classes as recommended by The National Antimicrobial Resistance Monitoring System and CLSI guidelines, including antibiotics used in industrial agriculture or in human medicine with risk assessment priority levels based on The World Health Organization criteria. (The Centers for Disease Control and Prevention, 2016; Clinical Laboratory Standards Institute, 2014; U.S. Food and Drug Administration, 2012 & 2015; The World Health Organization, 2017).

Data and Statistical Analysis

Mean *E. coli* per 100 mL was calculated for each sample type, sample event and for all background sites and poultry sites. Sample concentrations were calculated by counting *E. coli* colonies of the most countable plates (20-80 colonies) then calculating the proportionate number per 100 mL. Then, the concentrations per sample type and sample event were averaged. 95% confidence intervals were calculated for each mean. Tableau® software was used to generate figures of 95% confidence intervals.

Precipitation data was collected using National Oceanic Atmospheric Administration's (NOAA) Advanced Hydrologic Predictive Service. Precipitation data was aggregated from NOAA data for 2 days and 1 day prior to each sampling event and during sampling event (U.S. Department of Commerce et al., 2018). ArcGIS was used to extract precipitation data for each sampling location. Antecedent precipitation to the sampling event graphs were recorded from USGS 02111391 near Wilkesboro, NC, with the USGS gauge being located 35-40 kilometers to the sampled watersheds (Figure 5) (U.S. Geological Survey, 2018).

Stream discharge was recorded on the sampling date from USGS 02112250 Yadkin River at Elkin, NC, the USGS gauge located closest to the sampled watersheds, with the closest sampled site being around 24 kilometers from the gauge and the farthest sampled site being around 42 kilometers from the gauge (U.S. Geological Survey, 2018).

GraphPad Software was used to run unpaired Welch t-tests between background and poultry samples and Microsoft Excel's Data Analysis package used for a simple linear regression model to examine how precipitation data and subsequently, stream discharge affect *E. coli* concentration. A multiple linear regression that included watershed area and precipitation to explain variations in *E. coli* was also conducted.

CHAPTER 5: RESULTS

E. coli concentration in background and poultry sites

The highest mean *E. coli* concentrations for both background (BK) and poultry (P) sites were observed on the first sampling date on 10/24/17, with concentrations at BK sites averaging 210 CFU/100 mL (95% CI: [-124,554]) and concentrations at P sites averaging 813 CFU/100mL (95% CI: [199,1427]) (Figure 3 & Table 1). The mean *E. coli* concentrations on the other three sample days for both sample types were much lower {BK = 85 CFU/100 mL (95% CI: [-50,196]), P = 134 CFU/100 mL (95% CI: [42,226} (Figure 3 & Table 1). *E. coli* concentration widely varied between sample type and site. Variation in mean *E. coli* concentrations were observed among sites, with sites P1 and P2 (627, 459 CFU/100 mL; 95% CI: [80,1173], [-684,1601] respectfully) showing highest observed *E. coli* concentrations (Figure 4 & Table 2). P1 had the highest mean concentration of *E. coli* of all poultry sites and background sites, with a mean concentration of 627 CFU/100 mL compared to other poultry sites with means that ranged from 281 to 459 CFU/100 mL and background sites that ranged from 46 to 380 CFU/100 mL. A Welch t test comparing concentration differences between background and poultry samples revealed a two tailed P value equal to 0.07, so the difference in concentration was not quite statistically significant ($P > 0.05$). The lack of significance is likely explained by a low number of samples and high variability of concentrations observed within samples.

Prevalence of antibiotic resistant *E. coli* in background and poultry sites

A total of 165 *E. coli* isolates were archived from sites with up to five isolates collected for each site for each sample time. A total of 165 isolates were tested for antibiotic resistance, and four of those were found to be resistant (2%) (Table 3). Two resistant isolates originated from poultry sites, with both isolates showing resistance to tetracycline. The other two resistant isolates originated from background

sites with one isolate resistant to tetracycline and the other isolate showing multi-drug resistance, with resistance to ampicillin, amoxicillin-clavulanate acid, ceftriaxone, cefoxitin and gentamicin.

Precipitation's effect on *E. coli* concentration

Average precipitation was highest on 10/24/17 (Figure 5 & Table 4, 48mm), and this rain event (event A) was the largest precipitation event with the highest stream discharge rate (86 m^3/s that was obtained from USGS 02112250 gauge on the Yadkin River at Elkin, NC) (Figure 7 & Table 5). The antecedent rainfall that occurred about 18-hours prior to the event contributed to the rising limb of the stream flow/discharge of this sampling event (Figure 8A). The largest amount of precipitation and highest stream discharge rate corresponds to the highest mean *E. coli* concentration (Figure 5 & Table 4, 545 CFU/100 mL) of all sample dates. This event also exemplifies a big difference between background and poultry *E. coli* concentration, with poultry (Table 1, 813 CFU/100 mL) having a greater mean concentration of *E. coli* compared to background (Table 1, 210 CFU/ 100 mL). Precipitation event B on 11/14/17 was a smaller rain event compared to A, and the mean concentrations of both background and poultry *E. coli* decreased. There was a large drop-off in stream discharge between the first (event A, 86 m^3/s) and second event (event B, 36 m^3/s), which directly corresponds to the mean concentration of *E. coli* dropping between the two events as well. Sampling event 11/17/17 was closest to a baseline level, as no two-day antecedent precipitation occurred (zero mm of precipitation) and it had the lowest stream discharge (26 m^3/s) compared to the other three events. Overall, rainfall had an effect size of 9.97 according to the regression; that is, for every 1 mm increase in rainfall, the concentration of *E. coli* increased by 9.97 CFU/100 mL. USGS 02111391 gauge's precipitation graphs (Figure 6) displayed the two-day antecedent rainfall of and during the time of sampling, which confirmed the first three sampling events occurred during peak rainfall events of this sampling period, and the last sampling event was classified as a baseline level as zero mm of precipitation occurred during that sampling period.

Area's effect on *E. coli* concentration

The watershed area of the nine different watersheds varied from 1.29 km^2 to 37.08 km^2. From a simple relationship graph, it appeared that an increase in area did not correlate with greater *E. coli* concentrations, except for site P1, which had the greatest area (37.08 km^2) and the greatest *E. coli* concentration (627 CFU/100 mL) (Figure 9). However, a simple linear regression did show a positive correlation between area and *E. coli* concentration (Figure 10). A multiple linear regression (MLR) analysis concluded that precipitation did have an impact in predicting higher *E. coli* concentrations (X1), and that it did matter whether the watershed was a background or poultry site (X3), as these coefficients were positive for the model: Y = -87.26 + 9.96(X1) -6.59(X2) + 369.16(X3). Area (X2) did not have a positive effect in predicting *E. coli* concentration in addition to precipitation based on this multiple linear regression analysis. Results of the MLR indicate that when controlling for precipitation and area, presence of poultry CAFO results, on average, resulted in higher *E. coli* concentrations compared to background sites by 369 CFU/100mL. When controlling for watershed type (presence of poultry) and area, every 1 mm increase in two-day antecedent precipitation results, on average, resulted in higher *E. coli* concentrations by 9.96 CFU/100mL (Table 7 & 8).

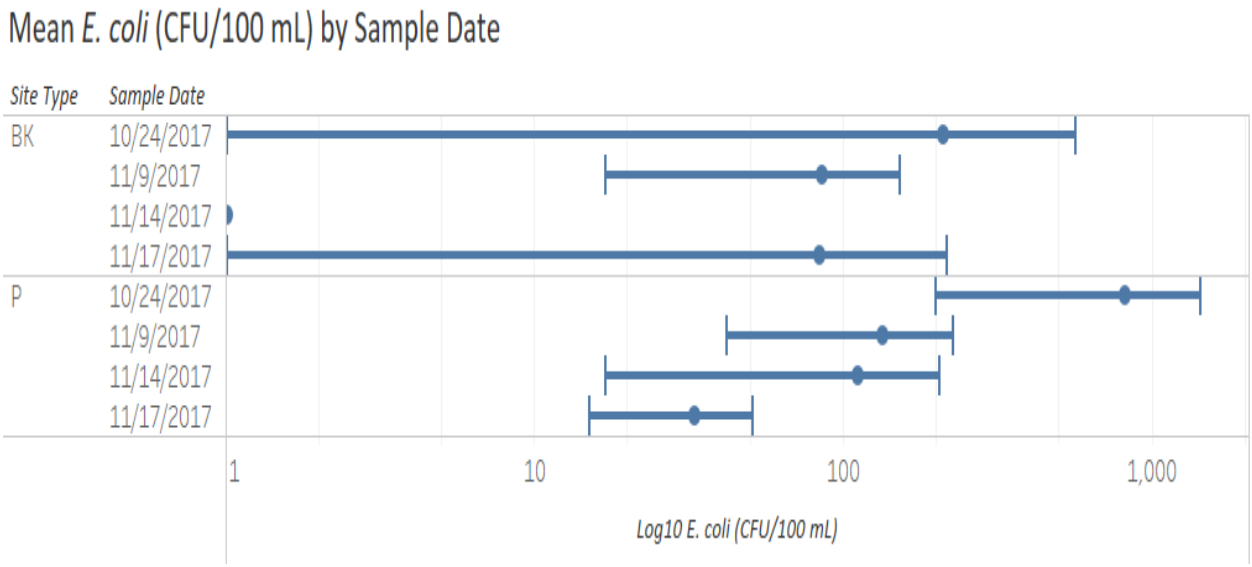

Figure 3. Mean *E. coli* concentrations (CFU/100 mL) on each sample date. Wings indicate 95% confidence intervals (CI). If lower CI was <0, the lower limit was set to 1.01 (log scale). BK refers to 'background site' (no poultry CAFO upstream). P refers to 'poultry site' (with a poultry CAFO upstream). 11 samples (11/36) had *E. coli* counts that were either below or above the limit of detection (20 – 80 colonies per plate). Sample date 11/9/17 had 3/4 BK and 0/5 P samples above/below the limit of detection, sample date 11/14/17 had 4/4 BK samples and 1/5 P samples above/below the limit of detection, sample date 11/17/17 had 1/4 BK and 2/5 P samples above/below the limit of detection. *E. coli* concentration is log scale

Table 1. 95% Mean *E. coli* concentrations (CFU/100 mL) on each sample date. BK refers to 'background site' (no poultry CAFO upstream). P refers to 'poultry site' (with a poultry CAFO upstream). 11 samples (11/36) had *E. coli* counts that were either below or above the limit of detection (20 – 80 colonies per plate). Sample date 11/9/17 had 3/4 BK and 0/5 P samples above/below the limit of detection, sample date 11/14/17 had 4/4 BK samples and 1/5 P samples above/below the limit of detection, sample date 11/17/17 had 1/4 BK and 2/5 P samples above/below the limit of detection.

Sample Date	Site Type	Description of Sampling Event	Mean *E. coli* (CFU/100 mL)	95% CI
10/24/2017	BK (n=4)	Large precipitation event A; peak rainfall of sampling period	210	(-124, 554)
11/9/2017	BK (n=1)	Smaller precipitation event B; peak rainfall of sampling period	85	(-50, 196)
11/14/2017	BK (n=0)	Smaller precipitation event B; peak rainfall of sampling period	< 20	N/A
11/17/2017	BK (n=3)	Close to baseline after precipitation event B; zero two-day antecedent rainfall	84	(-48, 216)
10/24/2017	P (n=5)	Large precipitation event A; peak rainfall of sampling period	813	(199, 1427)
11/9/2017	P (n=5)	Smaller precipitation event B; peak rainfall of sampling period	134	(42, 226)
11/14/2017	P (n=4)	Smaller precipitation event B; peak rainfall of sampling period	111	(17, 205
11/17/2017	P (n=3)	Close to baseline after precipitation event B; zero two-day antecedent rainfall	33	(15, 51)

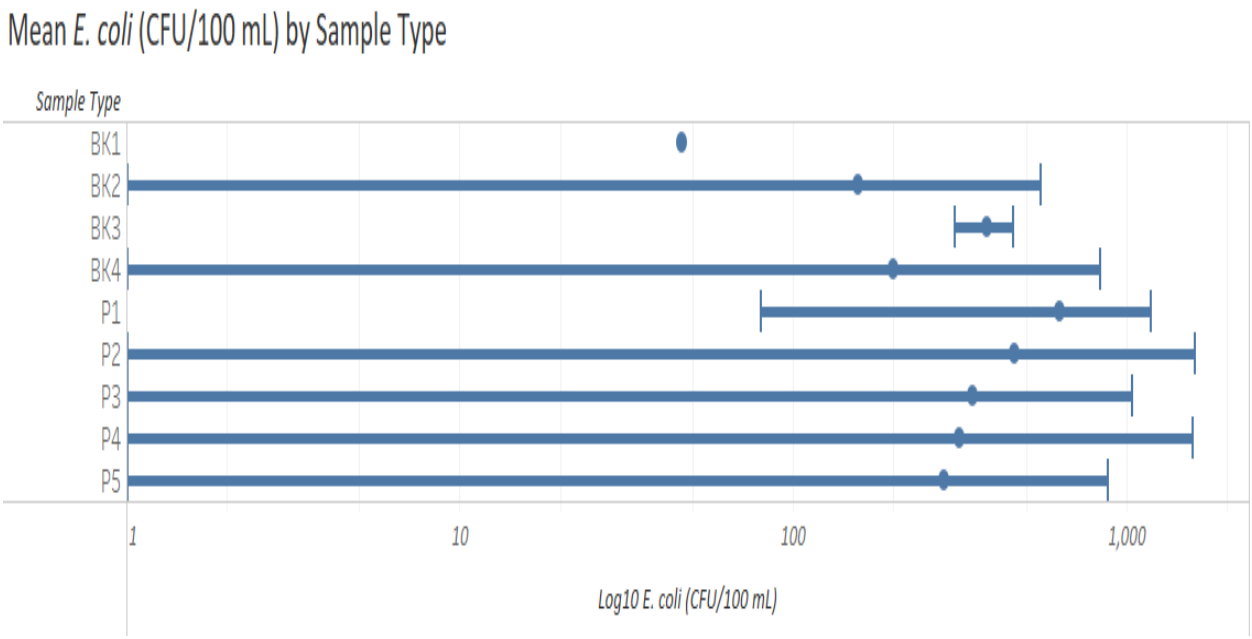

Figure 4. 95% Mean *E. coli* concentrations (CFU/100 mL) of each sample site. Wings indicate 95% confidence intervals (CI). If CI <0, the lower limit was set to 1.01 (log scale). BK refers to 'background site' (no poultry CAFO upstream). P refers to 'poultry site' (with poultry CAFO upstream). 11 samples (11/36) had *E. coli* counts that were either below or above the limit of detection (20 – 80 colonies per plate). *E. coli* concentration is log scale.

Table 2. 95% Mean *E. coli* concentrations (CFU/100 mL) of each sample site. BK refers to 'background site' (no poultry CAFO upstream). P refers to 'poultry site' (with poultry CAFO upstream).

Sample Type	Mean *E. coli* (CFU/ 100 mL)	95% CI
BK1 (n=1)	46	N/A
BK2 (n=2)	156	(-238, 550)
BK3 (n=2)	380	(304, 456)
BK4 (n=3)	198	(-435, 831)
P1 (n=3)	627	(80, 1173)
P2 (n=4)	459	(-684, 1601
P3 (n=4)	343	(-348, 1034)
P4 (n=2)	314	(-943, 1571)
P5 (n=4)	281	(-315, 877)

Table 3. Antibiotic resistance in *E. coli* isolates.

	Total Isolates Tested for Resistance: 165	
Antibiotics Tested	Background (n=50)	Poultry (n=115)
Tetracycline	1 (2%)	2 (2%)
Ampicillin	1 (2%)	0 (0%)
Amoxicillin-Clavulanate Acid	1 (2%)	0 (0%)
Ceftriaxone	1 (2%)	0 (0%)
Cefoxitin	1 (2%)	0 (0%)
Gentamicin	1 (2%)	0 (0%)
Chloramphenicol	0 (0%)	0 (0%)
Ciprofloxacin	0 (0%)	0 (0%)
Impinenem	0 (0%)	0 (0%)
Levofloxacin	0 (0%)	0 (0%)
Trimethoprim-Sulfamethoxazole	0 (0%)	0 (0%)

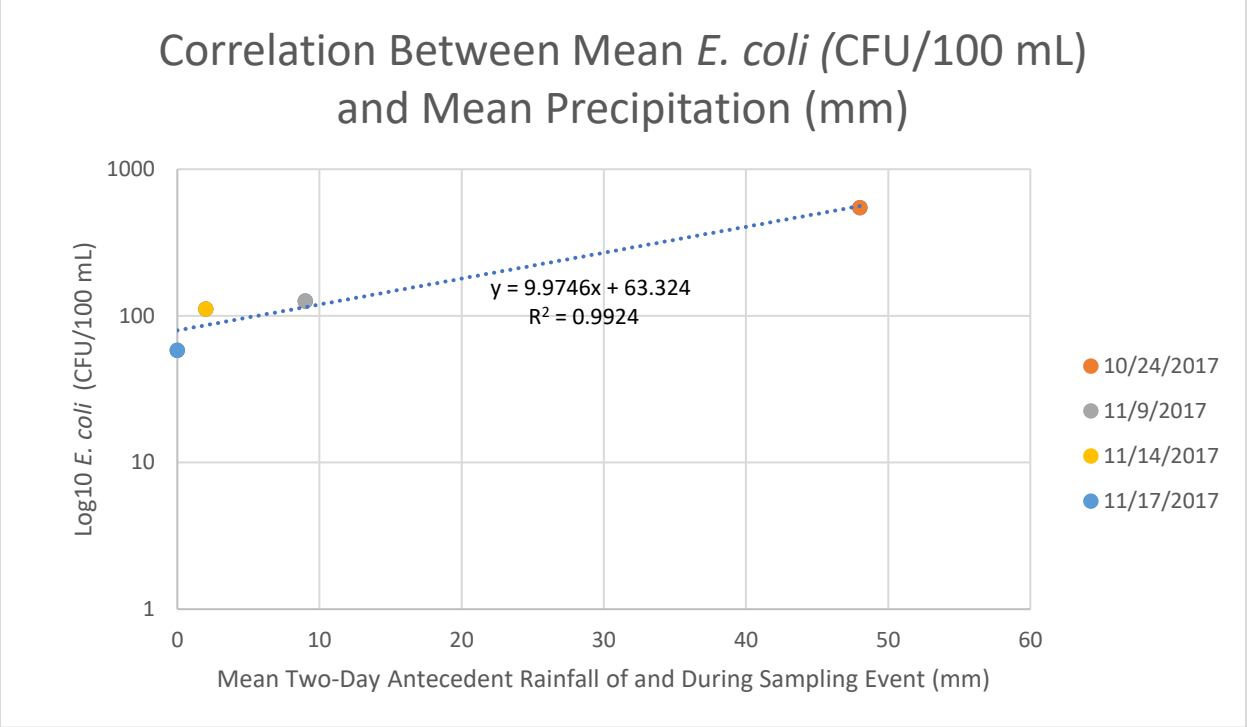

Figure 5. Simple linear regression between mean *E. coli* (CFU/100 mL) and precipitation (mm) on each sample date. Precipitation includes mean two-day antecedent rainfall prior to sample event and during sample event that was averaged between 9 samples on sample date. Data for mean *E. coli* (CFU/100 mL) per sample data was standardized, which excluded 11/36 samples. *E. coli* concentration is log scale.

Table 4. Mean two-day antecedent precipitation of and during sampling event, averaged across 9 sample locations for each sample date and its corresponding *E. coli* concentration.

Sample Date	Mean Two-Day Antecedent Rainfall of and During Sampling Event Across 9 Sample Sites (mm)	Mean *E. coli* (CFU/100 mL)
10/24/2017	48	545
11/9/2017	9	126
11/14/2017	2	111
11/17/2017	0	58

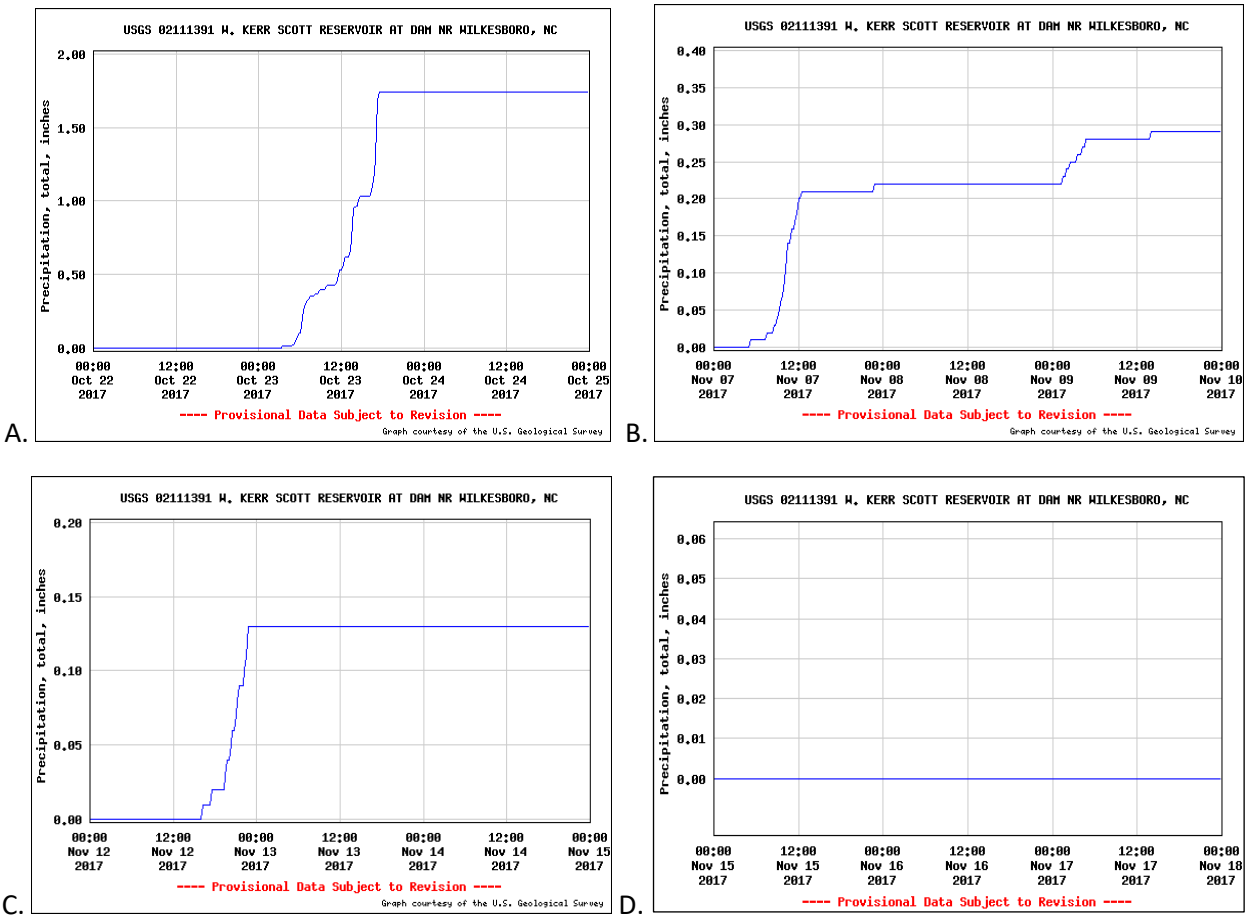

Figure 6. Recorded two-day antecedent rainfall (in) of and during sampling event from USGS 02111391 near Wilkesboro, NC. **A.** Sample date 10/24/17. Calculated average rainfall across the 9 sample sites was 1.89 in = 48 mm. **B.** Sample date 11/9/17. Calculated average rainfall across the 9 sample sites was 0.35 in = 9 mm. **C.** Sample date 11/14/17. Calculated average rainfall across the 9 sample sites was 0.079 in = 2 mm. **D.** Sample date 11/17/17. Calculated average rainfall across the 9 sample sites was 0 in = 0 mm.
Note: These graphs do not represent the actual aggregated precipitation data that was used for calculations but provide a visual of the overall rain events that occurred during each sampling event.

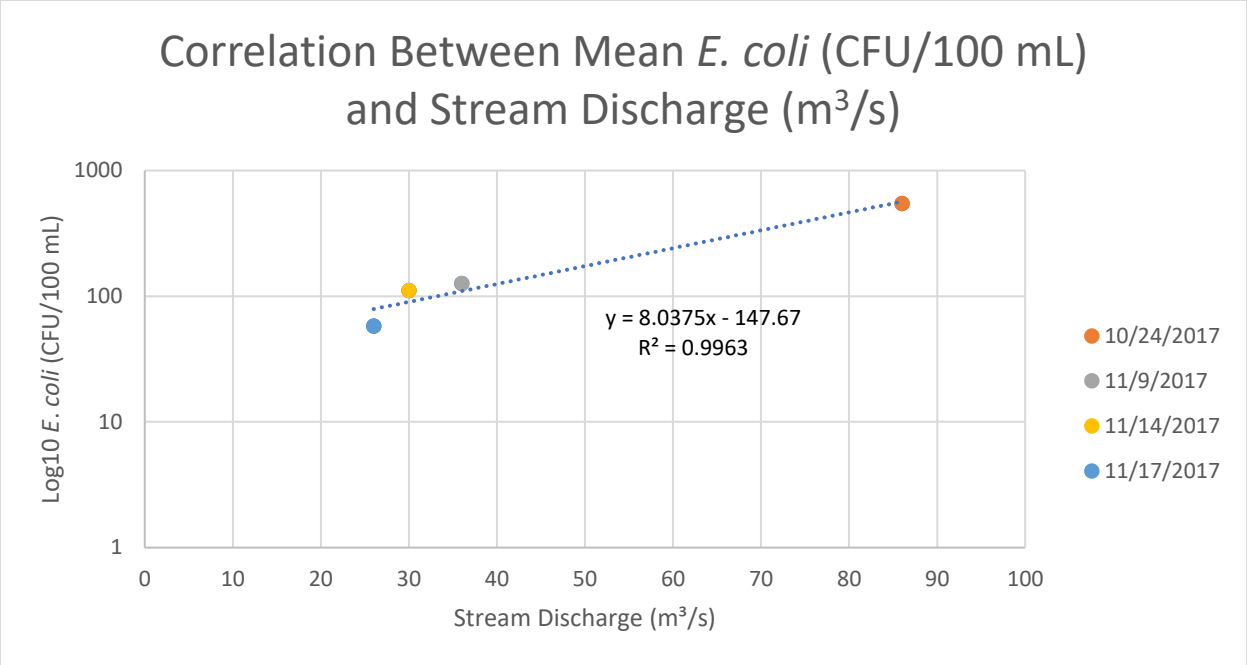

Figure 7. Simple linear regression between mean *E. coli* (CFU/100 mL) and stream discharge (m^3/s) from USGS 02112250 Yadkin River at Elkin, NC. *E. coli* concentration is log scale.

Table 5. Stream discharge (m^3/s) and its corresponding *E. coli* concentration on each sample date.

Sample Date	Stream Discharge (m^3/s)	Mean *E. coli* (CFU/100 mL)
10/24/2017	86	545
11/9/2017	36	126
11/14/2017	30	111
11/17/2017	26	58

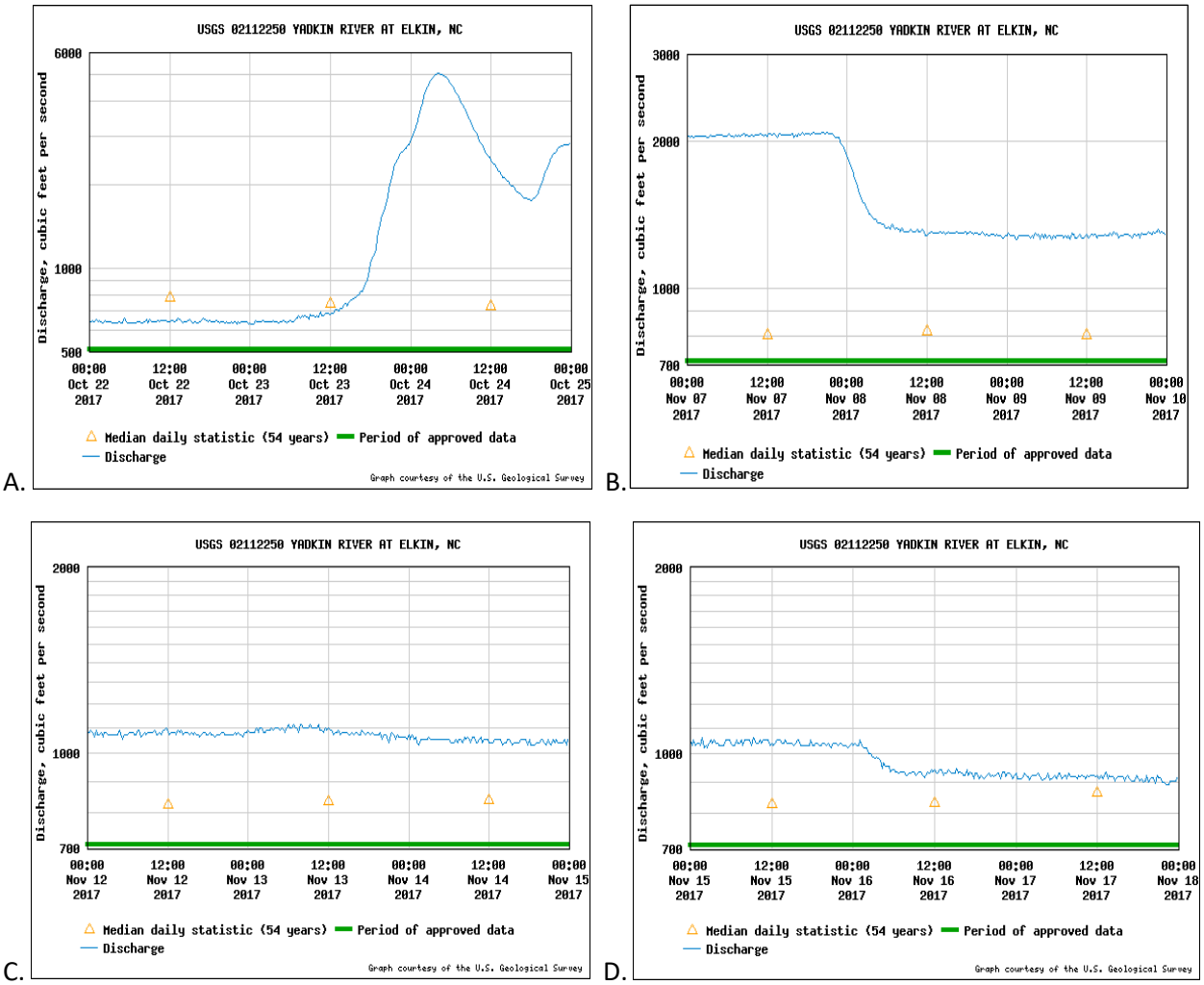

Figure 8. Hydrographs depicting two-day antecedent stream discharge (m^3/s) of and during sampling event, from USGS 02112250 Yadkin River at Elkin, NC. **A.** Sample date 10/24/17. Recorded stream discharge at time of sampling was 3040 ft^3/s = 86 m^3/s. **B.** Sample date 11/9/17. Recorded stream discharge at time of sampling was 1280 ft^3/s = 36 m^3/s. **C.** Sample date 11/14/17. Recorded stream discharge at time of sampling was 1050 ft^3/s = 30 m^3/s. **D.** Sample date 11/17/17. Recorded stream discharge at time of sampling was 914 ft^3/s = 26 m^3/s.

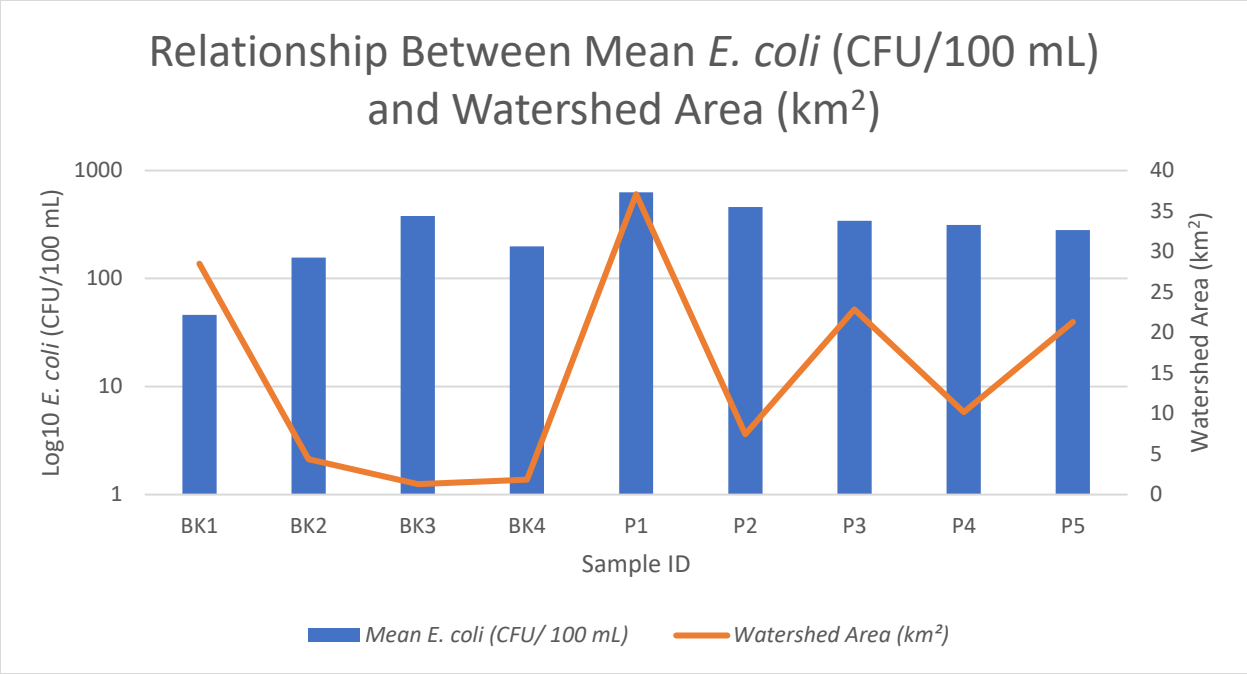

Figure 9. Relationship between mean *E. coli* (CFU/100 mL) and watershed area (km^2). *E. coli* concentration is log scale.

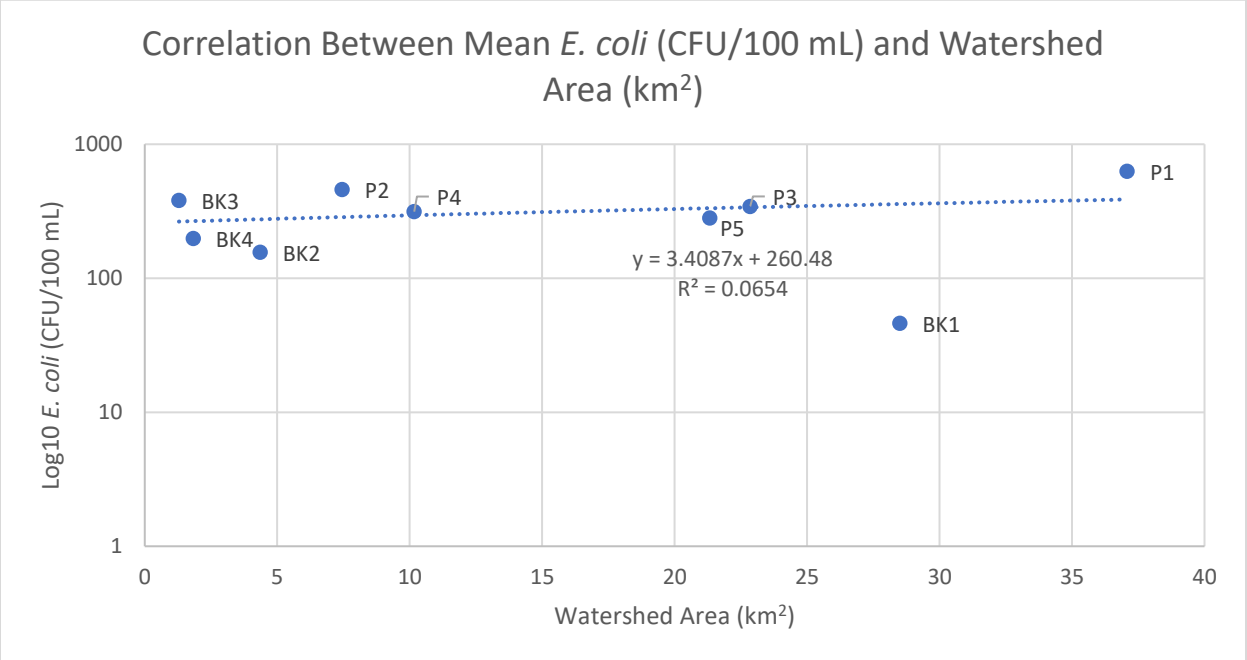

Figure 10. Simple linear regression between mean *E. coli* (CFU/100 mL) and watershed area (km^2). *E. coli* concentration is log scale.

Table 6. Predicted mean *E. coli* concentration by a multiple linear regression analysis. Variables included total two-day antecedent precipitation that included to time of sample event (mm) (X1), area (km^2) (X2), background or poultry sites (X3) and mean *E. coli* concentration (CFU/100 mL) (Y).

Sample Date	Site ID	X1: Total Two-Day Antecedent Precipitation (mm) of and During Sample Event	X2: Area (km^2)	X3: 0 for Background, 1 for Poultry	Y: *E. coli* (CFU/100 mL)	Predicted Y: *E. coli* (CFU/100 mL)
10/24/2017	BK1	48.5	28.5	0	46	327
10/24/2017	BK2	62.6	4.36	0	109	534
10/24/2017	BK3	44.1	1.29	0	196	366
10/24/2017	BK4	54.4	1.83	0	490	463
10/24/2017	P1	36.7	37.08	1	458	432
10/24/2017	P2	43.2	7.45	1	1530	583
10/24/2017	P3	44.1	22.85	1	980	546
10/24/2017	P4	55.2	10.17	1	256	690
10/24/2017	P5	44.1	21.32	1	840	550
11/9/2017	BK1	10.6	28.5	0	< 40	N/A
11/9/2017	BK2	12.2	4.36	0	< 40	N/A
11/9/2017	BK3	6.5	1.29	0	< 80	N/A
11/9/2017	BK4	8.7	1.83	0	85	25
11/9/2017	P1	7	37.08	1	129	147
11/9/2017	P2	8.2	7.45	1	87	248
11/9/2017	P3	6.5	22.85	1	252	185
11/9/2017	P4	10.7	10.17	1	58	263
11/9/2017	P5	6.5	21.32	1	143	190
11/14/2017	BK1	2.5	28.5	0	< 40	N/A
11/14/2017	BK2	1.9	4.36	0	< 20	N/A
11/14/2017	BK3	2	1.29	0	< 40	N/A
11/14/2017	BK4	1.8	1.83	0	< 40	N/A
11/14/2017	P1	2.3	37.08	1	40	102
11/14/2017	P2	3.4	7.45	1	200	202
11/14/2017	P3	2	22.85	1	110	142
11/14/2017	P4	2.6	10.17	1	< 20	N/A
11/14/2017	P5	2	21.32	1	92	147
11/17/2017	BK1	0	28.5	0	< 40	N/A
11/17/2017	BK2	0	4.36	0	47	-66
11/17/2017	BK3	0	1.29	0	184	-57
11/17/2017	BK4	0	1.83	0	20	-58
11/17/2017	P1	0	37.08	1	< 20	N/A
11/17/2017	P2	0	7.45	1	20	169
11/17/2017	P3	0	22.85	1	30	123
11/17/2017	P4	0	10.17	1	< 40	161

11/17/2017	P5	0	21.32	1	48	127

Table 7. Multiple linear regression analysis statistics.

Regression Statistics	
Multiple R	0.65
R Square	0.43
Adjusted R Square	0.35
Standard Error	291.98
Observations	25

Table 8. Multiple Linear Regression Fitted Model: Y = -87.26 + 9.96(X1) -6.59(X2) + 369.16(X3)

	Coefficients	*Standard Error*	*t Stat*	*P-value*
Intercept	-87.26	130.79	-0.67	0.51
X Variable 1: 48 Hour Antecedent Precipitation	9.96	2.73	3.64	$1.53x10^{-3}$
X Variable 2: Area	-6.59	6.04	-1.0904	0.29
X Variable 3: 0 for Background, 1 for Poultry	369.16	155.90	2.37	0.03

CHAPTER 6: DISCUSSION

Results of this study indicated a higher concentration of *E. coli* in surface waters with poultry concentrated animal feeding operations in the watershed compared to waters without poultry CAFOs. Every poultry site had on average higher numbers of *E. coli* compared to background sites. These results are consistent with previous studies reporting that CAFOs can leach larger amounts of *E. coli* into watersheds compared to background sites that lack the industry (Thurston-Enriquez et al., 2005, Hill et. al., 2005). It is important to note that these previous studies were "experimental plot" studies, meaning that conditions for these studies were better controlled and might not be like the ambient environmental conditions of this study. The differences in concentrations observed in this study were not statistically significant ($p > 0.05$). A study conducted by Rogers et al. in 2009 examined persistence of pathogenic bacteria and fecal indicator bacteria like *E. coli* in agricultural soil that contained poultry litter, which has implications for expanding this study to test soil surrounding the nine sample sites to compare *E. coli* concentration levels between soil and water, as the surrounding soils could contribute higher amounts of *E. coli*, especially during precipitation events in runoff. Another study has found that *E. coli* concentrations can be high (10^5 CFU/100 mL) in the sediment of the bodies of water (Crabill et al., 1999). Also, studies have found that the pathogen *Campylobacter jejuni* is highly prevalent among poultry, especially in warmer months, and can occur simultaneously with *E. coli*, meaning testing for the pathogen in the nine surface water samples could prove useful in also determining poultry CAFO contamination (Cox et al., 2002; Wills and Murray, 1997; USEPA, 2010).

There were few observations of antibiotic resistance among the *E. coli* isolated in these watersheds, let alone differences in between sample type. We did not find many isolates (4 out of 165) to be antibiotic resistant or to have multiple antibiotic resistance (1 out of 165). These young poultry

operations do not appear to be contributing widespread antibiotic resistance to the watersheds, but resistance elements should continue to be monitored, especially in the warmer seasons, if the operations persist in using antibiotics over time. A similar study also tested antibiotic resistance of *E. coli*, but tested actual fecal samples of turkeys, broilers, their famers and their slaughterers, found that there was statistically significantly higher ($p < 0.005$) antibiotic resistance to ciprofloxacin, flumequine and neomycin as compared to laying hens that didn't have high antibiotic usage, suggesting different sample types besides surface water, in addition to potentially supplementing different antibiotics, might need to be tested (Boggard, 2001). A related study also found high prevalence of antibiotic resistant *Staphylococcus aureus* in turkey (79%; 22/28) and chicken (26%; 6/23) isolates (Waters et al., 2011), also suggesting that other bacteria might need to be tested in addition to *E. coli* in this study to better quantify antibiotic resistance potential in the sample watersheds. This study's lack of finding antibiotic resistance greatly differs from studies that have tested for antibiotic resistance in watersheds that contain swine CAFOs. Sapkota et al. (2007) analyzed surface water samples that were downstream of a swine CAFO and found statistically significant antibiotic resistance of erythromycin ($p = 0.02$) and clindamycin ($p < 0.001$) in enterococci. A report by Christenson and Stewart (2018) also analyzed surface water samples that were downstream from swine CAFOs and found higher antibiotic resistance in swine sample *E. coli* compared to background sample *E. coli* (19% vs. 6%).

Three of the four sampling events took place during precipitation events. The first sampling event (precipitation event A) occurred during peak rainfall and contributed the highest amount of *E. coli* of both sample types from all sampling events, with poultry sites having more *E. coli* on average compared to background sites. This is likely due to a high stream discharge from the rain event, which would lead to increased concentrations of *E. coli* from the surrounding areas. There appears to be a positive trend (effect size = 9.97) as precipitation increases, *E. coli* concentration also increases. A multiple linear regression model {$Y = -87.26 + 9.96(X1) - 6.59(X2) + 369.16(X3)$} that included

precipitation (X1), area (X2), and whether the sample site was background or poultry (X3) found that precipitation (X1) did have an impact on predicting *E. coli* concentrations, as this coefficient was positive. Water quality impacts from poultry CAFOs are more likely to occur during and immediately after precipitation events, so precautions could be taken to buffer streams from runoff or to ensure that wastes are not land applied prior to a rain event (Hill et al., 2005; Dutta et al., 2010; Edwards and Daniel, 1993; Saurer et al., 1999). *E. coli* concentrations have been found to be high in the sediment of streams, rivers and lakes, and a previous study found increased *E. coli* concentrations into the water column due to resuspension of these sediments from rainstorms, meaning increased *E. coli* concentrations in the nine sample sites could also be attributed to resuspension of sediment particles, and not just an influx of contaminated runoff (Kim et al., 2010). This is consistent with research conducted by Cho et al. (2010) as they observed that there are sharp increases of fecal indictor bacteria after precipitation events from sediment.

It is interesting to note that the last sampling event on 11/17/17, the closest to a "baseline," had a higher mean CFU/ 100 mL in its background samples compared to the poultry samples (Figure 2 & Table 1). The difference between the two sample types' means was still greater than the mean poultry's concentration, possibly meaning that precipitation events lead to an increase in *E. coli* concentration from poultry CAFOs, but in the absence of a precipitation event, the background sites might contribute a greater amount, even with lower watershed areas. This could be due to various sources like human source pollution from septic systems or from wildlife (Ishii and Sadowsky, 2008). This could also be attributed to watershed area size, but the multiple linear regression model predicted that area does not have a positive impact on *E. coli* concentrations.

There were numerous limitations in this study including sampling location, seasonality, sample size and precipitation's dilution factor. It's possible that the study results could be affected by sampling locations within each watershed, as concentration of *E. coli* should increase with distance downstream

in a stream or watershed (Byappanahalli et al., 2003; Whitman et al., 1995). While the differences in *E. coli* numbers could be attested to difference in sample type or where the sample was taken, other contributors to differences in *E. coli* concentrations could be humans with failing, leaky septic tanks or other species of animals in the proximity of the watershed (Ishii & Sadowsky, 2008). Seasonality affects *E. coli* concentrations, and as this sampling was conducted in the late fall, this study might have missed antibiotic resistance that could have arisen in the summer as warmer temperatures lead to increased *E. coli* concentrations (Young & Thackston, 1999; Li et al., 2015). Also, lower temperatures in the fall could have caused *E. coli* to enter a non-culturable state, which means potential contamination could have been missed during this study's sampling time. (Perdek et al., 2003; United States Environmental Protection Agency, 2013).

To increase statistical power, more sampling events should be conducted, which would likewise increase the number of isolates subjected to antibiotic resistance testing. Increasing the sample size to around 50-100 samples would likewise lead to the multiple linear regression model having increased statistical power. Sampling and analysis should also be conducted in peak broiler production season in the late spring or summer (Koknaroglu et al., 2007; Pruit & Lavergne, 2013; United State Department of Agriculture, 2018). It is also possible a dilution effect occurred, because as area increases, there's likely more precipitation/water in the area, thus potentially decreasing concentration as per unit volume increases. Human fecal markers should also be tested in addition to the LA35 poultry fecal marker to rule out other sources of *E. coli* contamination. Distance of poultry CAFOs to sampling locations should also be considered.

Compared to other studies that only examined *E. coli* concentration and antibiotic resistance profiles in watersheds (Fincher et al. 2009) and studies that only examined storm water runoff and *E. coli* concentration (Harris et al., 2018; Chen & Chang, 2014), this study combines all three aspects of analyzing *E. coli* concentration, the bacteria's antibiotic resistance profiles, and precipitation and

provides more information of how precipitation events affect water quality in an inland watershed. This study suggests that poultry CAFOs may affect water quality as indicated by concentrations of *E. coli*, but that the operations in the tested watersheds do not appear to be disseminating antibiotic resistant bacteria. The poultry industry in general is leading efforts to curb antibiotic use in food animal production, which may explain differences observed in this study versus in studies conducted in watersheds downstream of hog CAFOs (Meyer, 2017; Christenson et al., 2018; Sapkota et al., 2007). Alternatively, impacts could be seasonal. Future studies should include sampling in the spring and during peak broiler production season and collecting soil and sediment samples. Together this work will facilitate the effort in maintaining safe, clean water for humans and the environment in western North Carolina.

CHAPTER 7: CONCLUSION

This research contributes data on effects of poultry concentrated animal feeding operations on water quality. We found higher concentrations of *Escherichia coli* in watersheds containing poultry CAFOs, but the difference was not quite statistically significant ($p = 0.07$). A simple linear correlation showed that there appears to be a positive correlation with higher *E. coli* concentration and increased precipitation (effect size = 9.97). A multiple linear regression that incorporated precipitation, watershed area and whether the sample site was poultry or background revealed that two-day antecedent rainfall and poultry presence has an impact on *E. coli* concentration, but area does not. Results of antibiotic resistant testing of isolated *E. coli* showed low levels of resistance and little difference between sample types.

Further research should focus on sampling in peak broiler production season in the spring and summer seasons, collect soil and sediment samples, test microbial source tracking markers such as poultry-associated LA35 and human-associated HF183, sample immediately after precipitation events, and collect more water samples for greater statistical power. This work combined with future work, particularly tests conducted in peak broiler production season, could inform regulatory bodies on policies related to antibiotic use, waste management, and water quality protection.

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